Interplay between phase I and phase II biotransformation of drugs

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Chapter 3

Reconstitution of the interplay between cytochrome P450s and human glutathione S-transferases in clozapine metabolism in yeast

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ABSTRACT

Clozapine, an often-prescribed antipsychotic drug, is implicated in severe adverse drug reactions (ADRs). Formation of reactive intermediates by cytochrome P450s (CYPs) has been proposed as a possible explanation for these ADRs. Moreover, a protective role for human glutathione S-transferases (hGSTs) was recently shown using purified enzymes. We investigated the interplay between CYP bioactivation and GST detoxification in a reconstituted cellular context using recombinant yeast expressing a bacterial CYP BM3 mutant (M11), mimicking the drug-metabolizing potential of human CYPs, combined with hGSTA1-1, M1-1 or P1-1. Clozapine and the N-desmethylclozapine metabolite caused comparable growth inhibition and reactive oxygen species (ROS) formation, whereas the clozapine-N-oxide metabolite was clearly less toxic. Clozapine metabolism by BM3 M11 and the hGSTs in yeast was confirmed by identification of stable clozapine metabolites and hGST isoform-specific glutathione-conjugates. Oxidative metabolism of clozapine by BM3 M11 increased ROS formation and growth inhibition. Co-expression of hGSTP1-1 protected yeast from BM3 M11 induced growth inhibition in presence of clozapine, whereas similar expression levels of hGSTA1-1 and hGSTM1-1 did not. ROS formation was not lowered by hGSTP1-1 co-expression and was unrelated to mitochondrial electron transport chain (mETC) activity. We present a novel cellular model to study the effect of CYP and GST interplay in drug toxicity.

INTRODUCTION

Clozapine is an atypical antipsychotic drug generally considered as one of the most effective drugs available for the treatment of schizophrenia (*1–3*). However, therapeutic use of clozapine is limited by the occurrence of severe adverse drug reactions (ADRs), including idiosyncratic hepatotoxicity, agranulocytosis and cardiac toxicity (*4–7*).

Although the precise mechanisms explaining these clozapine-induced ADRs remain to be elucidated, the formation of a reactive nitrenium ion is often proposed to play an important role (*8–11*). Clozapine undergoes extensive oxidative metabolism in the human body, of which N-demethylation and N-oxidation are the major reactions involved (*10*). Besides these stable metabolites, clozapine is bioactivated by cytochrome P450 (CYP) enzymes into a reactive nitrenium ion, with human CYP3A4 and CYP2D6 showing the highest activity (*12*). Several *in vitro* studies demonstrated that clozapine cytotoxicity is mediated by oxidative metabolism. Upon bioactivation of clozapine by horseradish peroxidase (HRP), apoptosis was induced in human neutrophils and a dose-dependent decrease in viability of human bone marrow stromal cells was observed (*11*, *13*). Expression of human CYP3A4 in a human hepatocyte derived cell line with low background CYP activity markedly increased clozapine toxicity (*14*). Clozapine-induced toxicity in rat hepatocytes was also shown to be CYP dependent, in particular CYP3A and CYP2E1 (*15*). This cytotoxicity was accompanied by an increased formation of reactive oxygen species (ROS). Cellular glutathione (GSH) levels were depleted after clozapine treatment and addition of exogenous GSH prevented clozapine-induced cytotoxicity, stressing a protective role for GSH (*13*, *15*).

The clozapine nitrenium ion can react with GSH to form distinct GSH conjugates as identified in human liver microsomes and in rat and mice *in vivo* (*8*, *9*). Although GSH conjugation may occur spontaneously, it is also catalyzed by various glutathione S-transferases (GSTs). Some of these GST isoforms are known to be polymorphic within the human population, which might affect disease- and drug susceptibility (*16*). By combining the purified bacterial CYP BM3 (CYP102A1) mutant M11, capable of bioactivating clozapine, with several recombinant cytosolic human GSTs (A1-1, M1-1 and P1-1), a protective role for some of these hGSTs against the reactive clozapine intermediate has been proposed (*17*). Isoform-specific GSH conjugates were formed by these three different hGSTs, which were not formed in a spontaneous reaction with GSH.

To explore the effects of both CYP and GST biotransformation in clozapine toxicity in a cellular context, we aimed to construct a model using the yeast *Saccharomyces cerevisiae*. This yeast is a model eukaryote popular for its genetic accessibility, genome-wide screens, cost-effectiveness and rapid growth (*18*). Its potential use to study biotransformation-related toxicity of drugs, and a comparison with other cellular models, has recently been reviewed (*19*). Yeast has several endogenous enzymes classified as GSTs, of which some have been described to play a role in the protection against oxidative- and xenobiotic-induced stress (*20*, *21*). However, their ability to catalyze the GSH conjugation of the chemically reactive clozapine nitrenium ion is unknown. Moreover, wild type yeast strains lack CYP-genes responsible in humans for bioactivation of drugs. We selected the bacterial CYP BM3 (CYP102A1) mutant M11 as a model. This mutant was engineered to mimic the drug-metabolizing potential of human CYPs, with high catalytic activity towards a broad range of drugs including clozapine (*22*). We previously used a similar yeast model expressing this CYP BM3 mutant to investigate metabolism related toxicity of the non-steroidal inflammatory drugs (*23*, *24*).

In this study we generated yeast expressing CYP BM3 M11, either alone or in combination with different human GST isoforms (A1-1, M1-1 and P1-1), to investigate the possible involvement and interplay of these metabolic enzymes in clozapine toxicity.

MATERIALS AND METHODS

Chemicals and stock solutions

All chemicals were of analytical grade and purchased from commercial suppliers. Clozapine (100 mM), clozapine-N-oxide and N-desmethylclozapine (25 mM) were dissolved in DMSO. 2′,7′-dichlorodihydrofluoresceindiacetate was dissolved in ethanol (8 mM). 1-Chloro-2,4-dinitrobenzene (CDNB) was dissolved in ethanol (20 mM). Stock solutions were stored at −20 °C and protected from light.

Yeast strains and growth conditions

The haploid *Saccharomyces cerevisiae* strain BY4741 (MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0) was obtained from EUROSCARF. Strains without mitochondrial DNA (rho^0) were generated as described previously (*25*). Strains were grown in selective minimal media (YNB: 0.67% yeast nitrogen base without amino acids, 2% glucose, supplemented with amino acids except leucine and/

or uracil) at 30 °C with orbital shaking.

Construction of CYP BM3 M11 and human GST expressing yeast strains

The CYP BM3 M11 (M11) gene was obtained from the previously described pET28-M11 plasmid (*22*) and cloned using standard methods behind a constitutively active phosphoglycerate kinase (PGK1) promoter in YEplac181, a yeast vector carrying a 2 μ origin of replication (ori) and *LEU2* selection marker (*26*). Coding regions of human GSTA1-1, M1-1 and P1-1 were amplified with PCR from *Escherichia coli* expression plasmids that were a kind gift from Prof. Mannervik (Department of Biochemistry and Organic chemistry, Uppsala University, Sweden). NotI and XbaI restriction sites were introduced respectively before and after the coding regions, and used for cloning behind a PGK1 promoter into YEplac195 (2 μ ori, *URA3*). The resulting plasmids and the empty YEplac181 or YEplac195 were transformed into BY4741 in several combinations by using the freeze-thaw method (*27*). Strains constructed in this study and their assigned names are described in Table 1.

Table 1. Transformed BY4741 strains constructed in this study.

Assigned name	Plasmid(s)
C	YEplac181 (empty)
M11	YEplac181-PGK1-M11
C+C	YEplac181 (empty) + YEplac195 (empty)
M11+C	YEplac181-PGK1-M11 + YEplac195 (empty)
M11+GSTA1-1	YEplac181-PGK1-M11 + YEplac195-PGK1-GSTA1-1
M11+GSTM1-1	YEplac181-PGK1-M11 + YEplac195-PGK1-GSTM1-1
M11+GSTP1-1	YEplac181-PGK1-M11 + YEplac195-PGK1-GSTP1-1
C+GSTP1-1	YEplac181 (empty) + YEplac195-PGK1-GSTP1-1

Determination of GST activity

Cells from overnight cultures were harvested by centrifugation (3 min, 2500 × *g*), suspended in 100 mM potassium phosphate buffer pH 6.5. Cell suspensions were lysed using glass beads. Cell-free extracts were assayed with CDNB (1 mM) and GSH (1 mM) in 100 mM potassium phosphate buffer pH 6.5 at 24 °C. Linear increase in absorbance at 340 nm was measured in a Tecan Infinite 200 platereader and used to calculate the activity using a molar extinction coefficient of 9.6 mM^{-1} cm^{-1} (*28*). Activity was corrected for the total protein concentrations in the extracts determined using the bicinchoninic acid method (Pierce BCA protein assay kit, Thermo Scientific).

Determination of cytochrome P450 BM3 M11 activity

Cells from overnight cultures were harvested by centrifugation and suspended in 100 mM potassium phosphate buffer pH 7.4 supplemented with 2% (w/v) glucose. Testosterone (0.5 mM) was added and cells were incubated for 1 h at 24 °C. Samples were drawn at 15 min intervals, mixed with 1 volume of ice-cold methanol and incubated for 10 min on ice. Precipitation was removed by centrifugation (10 min, 20,800 × *g*) and the supernatant was analyzed by ultra performance liquid chromatography (UPLC) using an isocratic method previously described (*29*). Metabolite formation was corrected for the amount of cells (OD_{600}) in the samples.

Growth assays with clozapine, clozapine-N-oxide and *N*-desmethylclozapine

Overnight cultures were diluted to an optical density (OD_{600}) of 0.1 in selective minimal media buffered to pH 7.0 with 3-(N-morpholino)propanesulfonic acid (MOPS, 165 mM). Cultures were transferred to 48-well microtiter plates and incubated in a Tecan Infinite 200 platereader (30 °C, orbital shaking). Data was collected using Tecan Magellan 7 software. After 3 h preincubation, clozapine, clozapine-N-oxide or N-desmethylclozapine was added from DMSO stock solutions. Controls were treated with equal amounts of DMSO (1%, v/v). Growth was resumed while measuring the OD_{600} every 20 min for up to 72 h. Data was exported to Graphpad Prism 4 and the area under curve (AUC) was calculated.

In vivo generation of clozapine metabolites in yeast

Overnight cultures were diluted to an OD_{600} of 0.2 in 25 mL buffered selective minimal media (pH 7.0) and grown at 30 °C. After 3 h clozapine was added to a final concentration of 200 µM, and incubation was continued for 48 h. Cells were harvested, washed, suspended in 100 mM potassium phosphate buffer pH 7.4 and lysed using glass beads. Proteins were precipitated by adding 0.1 volume of 10% perchloric acid and removed by centrifugation (10 min 20,800 × *g*). Incubation media and cell-free extracts were passed through a Phenomenex Strata X33reversed phase SPE column, washed and metabolites were eluted with methanol. Eluates were dried under nitrogen and resuspended in a small volume of 50% methanol. Samples were analyzed by HPLC-MS as described previously (*17*). Data was analyzed using Agilent MassHunter Qualitative analysis software.

Measurement of ROS formation

Overnight cultures were diluted to a optical density (OD_{600}) of 0.2 in selective minimal media and grown for 3 h at 30 °C. 2′,7′-dichlorodihydrofluoresceindiacetate was added to a final concentration of 10 µM and incubation was continued for another hour at 30 °C. Cells were harvested by centrifugation (3 min 3800 × *g*) and washed with ice-cold 100 mM potassium phosphate buffer pH 7.0 supplemented with 2% glucose (w/v). Cells were suspended in the same buffer heated to 30 °C, transferred to a 96 well microtiter plate (black with clear bottom) and either clozapine, clozapine-N-oxide, N-desmethylclozapine, or equal amount of DMSO was added. Fluorescence (λ_{ex} 485 nm, λ_{em} 535 nm) and OD_{600} was measured every 15 min in a Tecan Infinite 200 platereader (30 °C, orbital shaking). Fluorescence was corrected for cell densities in the samples, yielding relative fluorescent units (RFU).

Statistical analysis

All experiments were performed at least twice, with at least two independent yeast cultures of each strain. Statistical analysis was performed in Graphpad Prism 4 using a two-sided unpaired *t*-test or using two-way ANOVA with Bonferroni post-test. P values < 0.05 were considered significant.

RESULTS

Co-expression of CYP BM3 M11 and hGST A1-1, M1-1 and P1-1 in yeast

Bioactivation of clozapine into the reactive nitrenium ion was aimed at in yeast by using the YEplac181-PGK1-M11 multicopy vector, which drives the expression of CYP BM3 M11 from a constitutive PGK1-promoter. Subsequent enzymatic GSH conjugation of the clozapine nitrenium ion was reconstituted by transforming the BM3 M11 strain with three different plasmids (YEplac195-PGK1) carrying human GST isoforms (A1-1, M1-1 or P1-1), or with a control plasmid to study non-enzymatic GSH conjugation. BM3 M11 activity in these co-expressing strains was confirmed and quantified by measuring testosterone hydroxylation in the cells, which appeared to be similar between the different strains (Fig. 1). hGST expression was calculated using the CDNB activity measured in the cell lysates and the specific activities previously reported for the different hGST isoforms (*17*). Expression levels (Fig. 2) were similar for all three hGST co-expressing strains (M11+GSTA1-1, M11+GSTM1-1, M11+GSTP1-1). The control strain (M11+C, data not shown) did not show significant CDNB conjugation in our assay, despite the presence of endogenous yeast GST-like enzymes previously reported to show low CDNB activity (*21*).

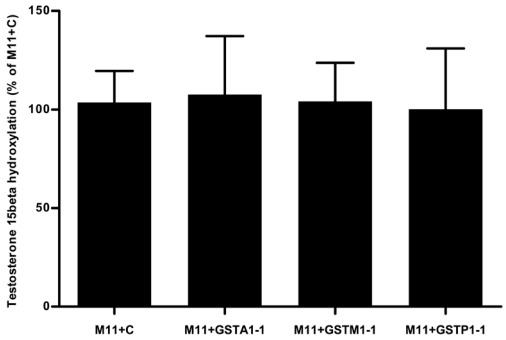

Fig. 1. Testosterone hydroxylation in yeast strains M11 expressing strains, without (M11+C) and with hGST co-expression (M11+GSTA1-1; M11+GSTM1-1; M11+GSTP1-1). Shown is the formation of 15-beta-hydroxytestosterone in whole cell incubations, expressed as percentage ± SD (n = 4) of the activity in the M11+C strain.

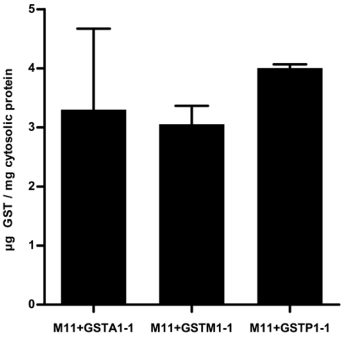

Fig. 2. Expression of human GST isoforms in M11 co-expressing yeast strains. Expression (mean µg GST/mg cytosolic protein ± SD, n = 3) was calculated based on the CDNB activity measured in yeast cellular extracts and the known specific activity for each isoform (*17*).

Clozapine metabolism in yeast co-expressing M11 with human GSTs

HPLC analyses of cellular extracts prepared from yeast strains incubated with clozapine (200 µM) were performed for the BM3 M11 expressing strain without (M11+C) and with hGSTP1-1 (M11+GSTP1-1), and for the wild type control (C+C). The chromatogram in Fig. 3 shows the N-desmethylclozapine (C-2, m/z313.13) as the major stable metabolite in all three strains tested and its formation was independent of hGST expression, but clearly induced by expression of BM3 M11. A second stable metabolite, clozapine-N-oxide (C-1, m/z 343.14) was much less abundant. Interestingly, both C-1 and C-2 were also detected in the wild type strain, though at low levels, perhaps due to endogenous yeast peroxidase activity. Two non-enzymatically formed GSH conjugates

(CG-1: C-6 glutathionyl clozapine, *m*/*z* 632.21; and CG-3: C-9 glutathionyl clozapine, *m*/*z* 632.21) were only present in the lysates of the BM3 M11 expressing yeast, confirming the formation of the reactive nitrenium ion. The formation of an additional conjugate (CG-6: C-8 glutathionyl deschloroclozapine, *m*/*z* 598.25) was dependent on hGSTP1-1 co-expression.

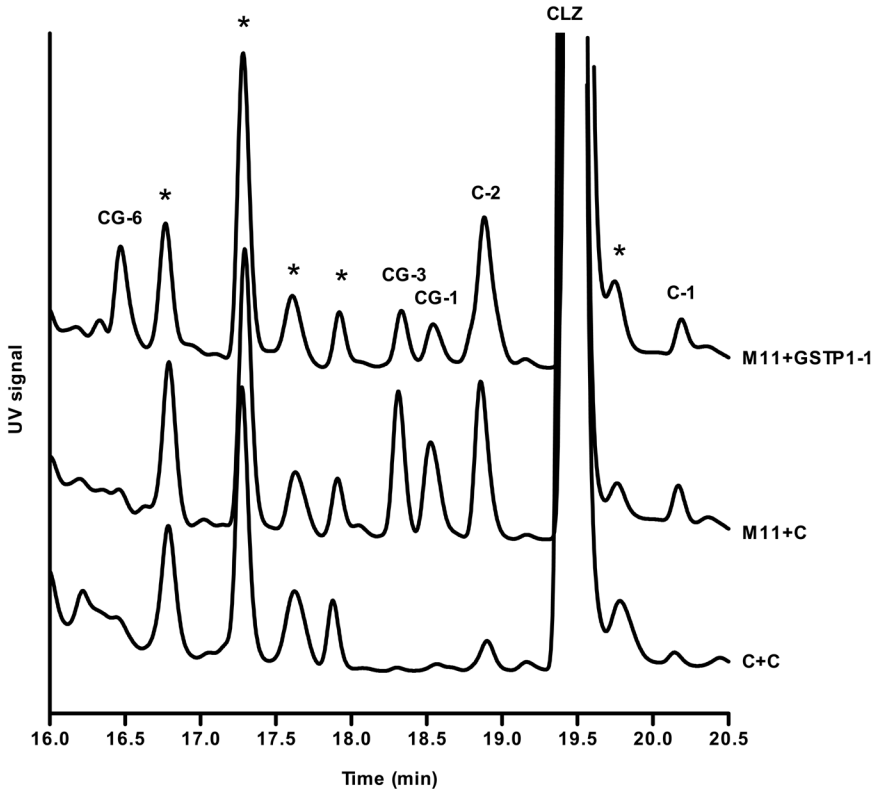

Fig. 3. Clozapine metabolites, stable and glutathione-conjugates, formed in yeast strains expressing M11. Shown are HPLC-UV chromatograms of yeast cellular extracts of yeast cells from the wild type (C+C), BM3 M11 expressing strain (M11+C), and BM3 M11 with hGSTP1-1 co-expressing strain (M11+GSTP1-1), each incubated with 200 μM clozapine. CLZ: clozapine; C-1: clozapine N-oxide; C-2: N-desmethylclozapine; CG-1: C-6 glutathionyl clozapine; CG-3: C-9 glutathionyl clozapine; CG-6: C-8 glutathionyl deschloroclozapine. Background peaks unrelated to clozapine metabolism are indicated by an asterisk (*). CG-5 (C1-4 glutathionyl clozapine) could only be detected by LC-MS due to coelution with C-2.

More detailed LC–MS analysis of all co-expression strains showed the formation of multiple clozapine GSH conjugates, previously shown to be present also in incubations with human liver microsomes (*17*, *22*) and *in vivo* in rats, mice and humans (*9*, *30–32*). Fig. 4 shows the relative abundance of these metabolites for the different strains. In the cellular extracts, five different clozapine GSH conjugates (CG-1; CG-3; CG-4: C-7 glutathionyl clozapine, *m*/*z* 632.21; CG-5: C1-4 glutathionyl clozapine, *m*/*z* 632.21; and CG-6) were detected by LC–MS and were identified by their masses, MS/MS product ions and retention times as described previously (*17*). Isoform-specificity of the hGST-catalyzed conjugates in cellular incubations was consistent with previous *in vitro* results with

pure enzymes (*17*). CG-4 was exclusively formed in the M11+GSTM1-1 co-expressing strain, and CG-5 only in the M11+GSTP1-1 strain (supplementary Fig. S1). CG-6 was also detected in small amounts in the BM3 M11 strain without GST, while it was previously reported to be absent in the non-enzymatic conjugation. The non-enzymatic CG-1 and CG-3 conjugates were formed in all four BM3 M11 expressing strains. While CG-1 was described to be clearly more abundant than CG-3 when clozapine was incubated with isolated BM3 M11, the yeast incubations showed a more equal ratio between the two conjugates, as judged by their HPLC-UV trace (Fig. 3). These differences in regioselectivity for the GSH conjugation might be the result of a much higher intracellular GSH concentration in yeast (reported up to 10 mM) (*33*) compared to the *in vitro* incubations with pure enzymes (100 μM), or possibly caused by the involvement of endogenous yeast GST-like enzymes.

Interestingly, five cysteine conjugates were identified, supposedly derived from the corresponding GSH conjugates after degradation in the yeast cells. Their structures are shown in Fig. 5. Four cysteine conjugates with a protonated molecular ion $[M+H]^+$ of *m*/z 446.15 were assigned to the corresponding GSH conjugates: CCys-1 (C-6 cysteine clozapine), CCys-3 (C-9 cysteine clozapine), CCys-4 (C-7 cysteine clozapine) and CCys-5 (C1-4 cysteine clozapine). These cysteine conjugates were identified based on references produced by collecting the corresponding GSH conjugates and treating them with γ-glutamyltranspeptidase. The MS/MS spectra of these conjugates showed product ions matching a fragmentation pattern previously described for the clozapine N-acetylcysteine-conjugates (*34*) with *m*/*z* 359, 328, and 302. A fifth cysteine conjugate with a molecular ion of *m*/*z* 412.19 lacked the typical chlorine isotope pattern; MS/MS showed a similar fragmentation pattern though with the loss of the chlorine atom, giving product ions of *m*/*z* 325, 294 and 268. This metabolite was thus assigned CCys-6 (C-8 cysteine deschloroclozapine).

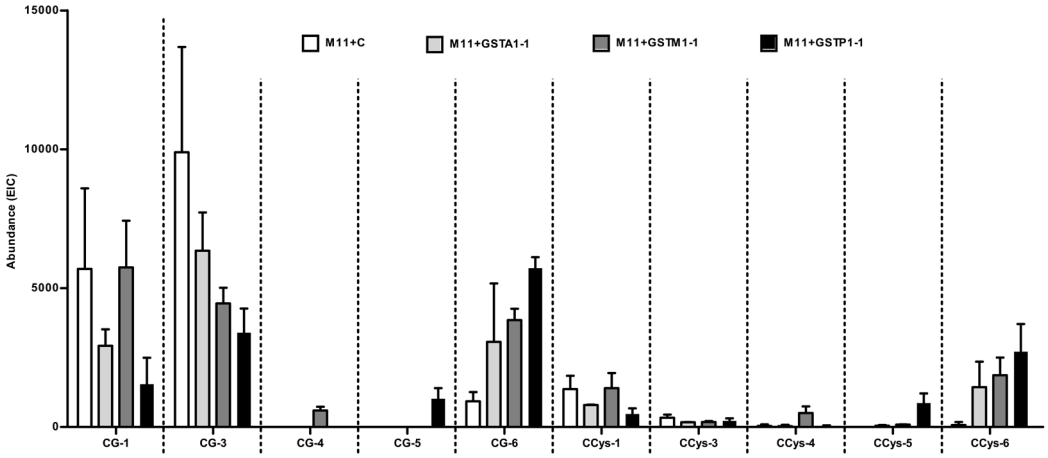

Fig. 4. Clozapine glutathione and cysteine conjugates formed in yeast strains expressing BM3 M11 without (M11+C, white bars) or with different hGSTs (M11+GSTA1-1, light grey bars; M11+GSTM1-1, dark grey bars; M11+GSTP1-1, black bars). Conjugates in cellular extracts of cells incubated with 200 μM clozapine were identified by LC–MS. Shown is the abundance of the conjugates, expressed as the mean peak area ± SD (n = 2) of the extracted ion chromatograms (EIC). N-desmethylclozapine formation was used as an internal standard. CG-1: C-6 glutathionyl clozapine; CG-3: C-9 glutathionyl clozapine; CG-4: C-7 glutathionyl clozapine; CG-5: C1-4 glutathionyl clozapine; CG-6: C-8 glutathionyl deschloroclozapine, CCys-1: C-6 cysteine clozapine; CCys-3: C-9 cysteine clozapine; CCys-4: C-7 cysteine clozapine; CCys-5: C1-4 cysteine clozapine; CCys-6: C-8 cysteine deschloroclozapine.

Toxicity of clozapine, N-desmethylclozapine and clozapine-N-oxide in yeast

Wild type yeast transformed with the two empty plasmids (C+C) was incubated with increasing concentrations of clozapine and its two stable metabolites, clozapine-N-oxide and N-desmethylclozapine (Fig. 6). Treatment with clozapine resulted in significant inhibition of growth. N-desmethylclozapine caused a similar decrease in growth, whereas clozapine-N-oxide was relatively non-toxic. Intracellular concentrations of the three compounds, as estimated by HPLC analysis of cellular extracts, showed a clearly lower concentration of clozapine-N-oxide (4.5 ± 0.7 nmol/mg cytosolic protein) compared to clozapine (10.7 ± 3.6 nmol/mg) and N-desmethylclozapine (9.3 ± 2.7 nmol/mg). Even considering the lower intracellular concentration of clozapine-N-oxide, the metabolite is causing significantly less inhibition of growth compared to the parent compound. These results are in line with those reported previously in human neutrophils, where clozapine and N-desmethylclozapine, but not clozapine-N-oxide, were shown to induce apoptosis without bioactivation (*11*).

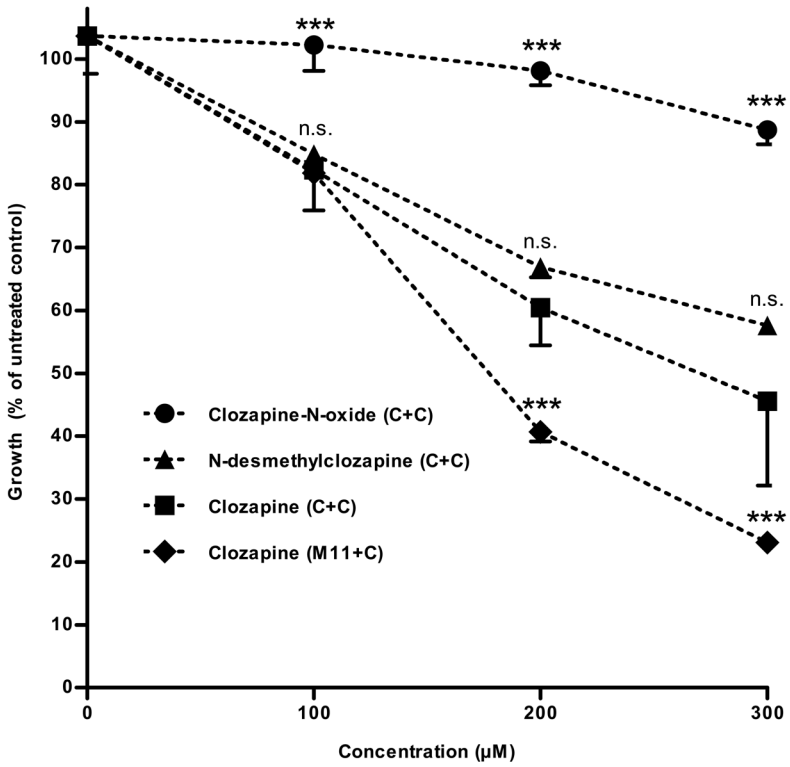

Fig. 6. Growth inhibition of wild type yeast (C+C) incubated for 21 h with clozapine (squares), clozapine-N-oxide (circles) and N-desmethylclozapine (triangles), and BM3 M11 expressing yeast (M11+C) with clozapine (diamonds). Growth is expressed as percentage growth ± SD ($n = 4$) compared to untreated cells. Dotted lines are intended only to guide the eye and do not represent fits to the data. Statistical analysis performed by two-way ANOVA compared to control strain (C+C) treated with clozapine for each concentration tested: n.s. not significant, ***$P < 0.001$.

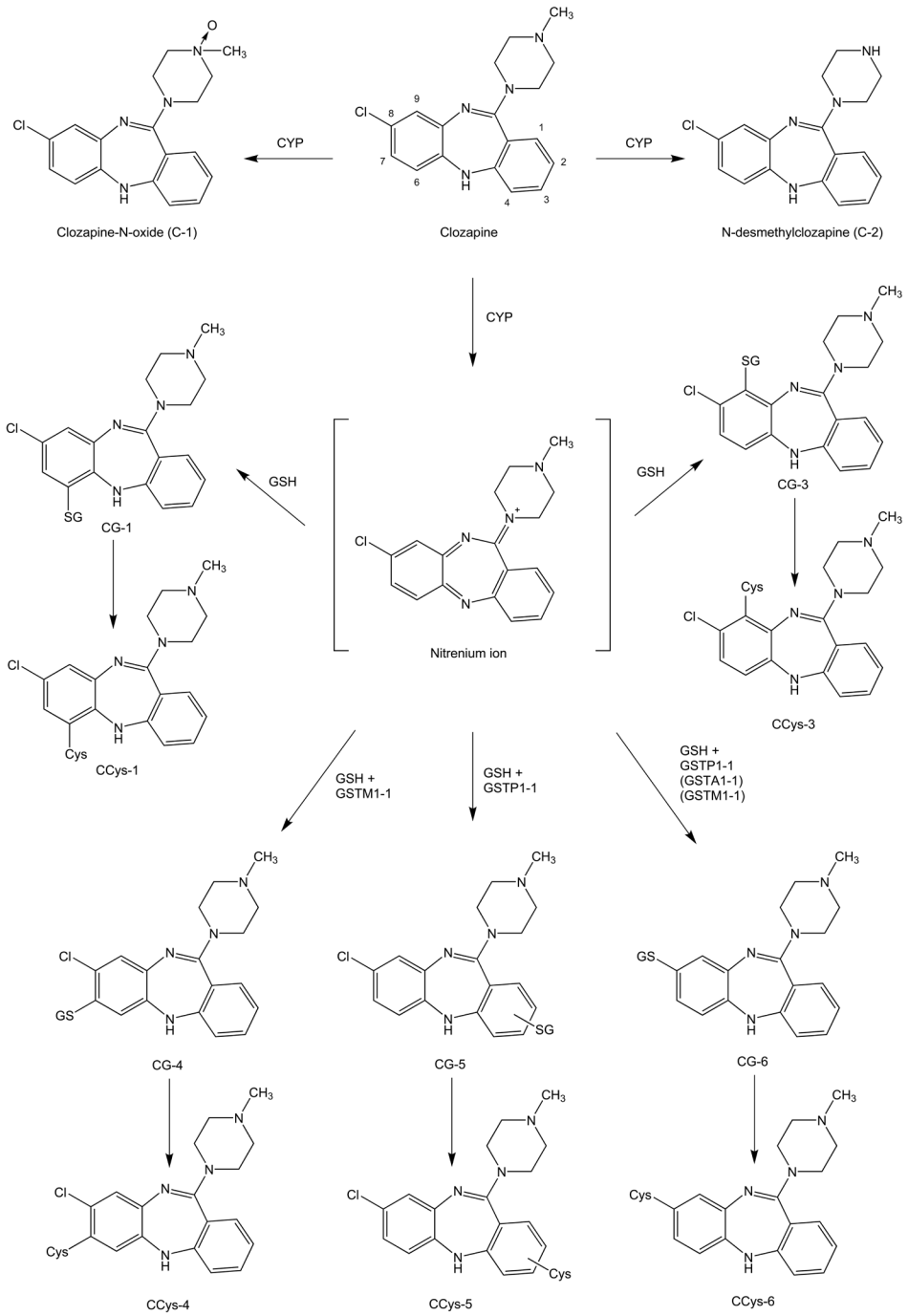

Fig. 5. Metabolic scheme of identified clozapine metabolites in yeast co-expressing BM3 M11 with different human GSTs. The proposed nitrenium ion is drawn between brackets. CG-1: C-6 glutathionyl clozapine; CG-3: C-9 glutathionyl clozapine; CG-4: C-7 glutathionyl clozapine; CG-5: C1-4 glutathionyl clozapine; CG-6: C-8 glutathionyl deschloroclozapine, CCys-1: C-6 cysteine clozapine; CCys-3: C-9 cysteine clozapine; CCys-5: C1-4 cysteine clozapine; CCys-6: C-8 cysteine deschloroclozapine.

Effect of co-expression of hGSTs on M11-dependent clozapine toxicity

The effect of both clozapine bioactivation and GSH conjugation on growth was studied in strains co-expressing BM3 M11 with three human GSTs. Compared to the control strain transformed with both empty plasmids (C+C), the BM3 M11 strain without GST (M11+C) showed a significant increase in growth inhibition after 48 h when incubated with 200 μM clozapine (Fig. 7). Strains co-expressing BM3 M11 with hGSTA1-1 (M11+GSTA1-1) or hGSTM1-1 (M11+GSTM1-1) still showed a significant decrease of growth compared to the control strain. In contrast, co-expression of BM3 M11 with hGSTP1-1 (M11+GSTP1-1) resulted in a protection against the BM3 M11 dependent increase in growth inhibition, as growth of this strain was no longer significantly different from the control.

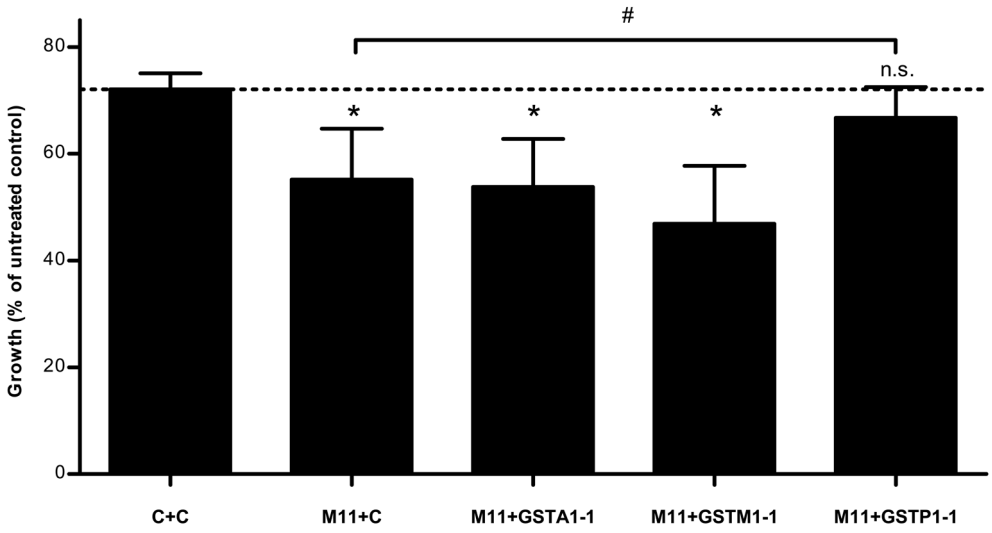

Fig. 7. Growth inhibition of co-expression strains after 48 h incubation with clozapine (200 μM). Shown are the control strain (C+C), the strains expressing BM3 M11 without hGST (M11+C) and the strains co-expressing the three different hGSTs with BM3 M11 (M11+GSTA1-1, M11+GSTM1-1 and M11+GSTP1-1). Growth is expressed as percentage growth ± SD ($n = 6$) compared to untreated cells. Statistical analysis performed by two-sided unpaired *t*-test, each bar compared to the control strain treated with clozapine (C+C) unless otherwise indicated by connecting lines: n.s. not significant; $^{\#}$,$^{*}P < 0.05$

Additional experiments were performed including a strain expressing hGSTP1-1 without BM3 M11 (C+GSTP1-1). These results (supplementary Fig. S2) ruled out a possible protective effect of hGSTP1-1 expression independent of clozapine bioactivation, as it was not able to protect against the toxicity of un-metabolized clozapine, while confirming the increased toxicity in the BM3 M11 strain (M11+C) and subsequent rescue by co-expression of hGSTP1-1 (M11+GSTP1-1).

Clozapine induces ROS formation in yeast

Clozapine can induce the formation of ROS in mammalian cells and the oxidation of proteins in humans (*15*, *35*). Therefore we also investigated the ROS formation in our yeast strains upon treatment with clozapine and the stable clozapine-N-oxide and N-desmethylclozapine metabolites using the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate as an approximate measure of intracellular ROS levels. Treatment of the wild type strain (C+C) with clozapine and the two stable metabolites all resulted in significant induction of ROS levels (Fig. 8a), although N-desmethylclozapine at a slightly higher initial rate, and clozapine-N-oxide reaching a somewhat lower level. The curves of clozapine-N-oxide and N-desmethylclozapine both show the formation of a plateau. Several factors may contribute to this effect, either alone or in combination. These factors could include a decreased ROS production in time, increased detoxification of ROS by adaptation of the yeast, induction of cytotoxicity, or possibly the degradation of the fluorophore.

Bioactivation of clozapine by BM3 M11 (M11+C) showed a modest but significant increase in ROS levels (Fig. 8b). Interestingly, co-expression of hGSTP1-1 (M11+GSTP1-1) further increased intracellular ROS, even though this co-expression showed protection against clozapine-induced growth inhibition in the BM3 M11 strain. Altogether, ROS formation could not directly be linked to the decrease in growth upon exposure to clozapine or its metabolites.

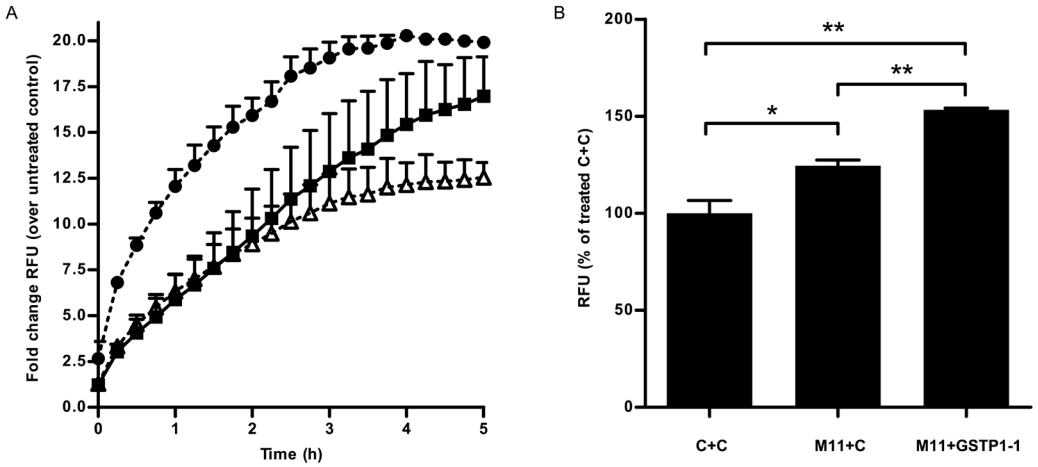

Fig. 8. Relative formation of ROS in yeast cells treated with clozapine, clozapine-N-oxide and N-desmethylclozapine (200 µM). (A) Exposure of wild type yeast (C+C) with clozapine (squares), clozapine-N-oxide (triangles) and N-desmethylclozapine (circles) expressed as fold change in RFU over untreated controls ± SD ($n = 2$). Lines are intended only to guide the eye and do not represent fits to the data. (B) Relative formation of ROS after 5 h incubation with clozapine in yeast expressing BM3 M11 (M11+C) or co-expressing BM3 M11 with hGSTP1-1 (M11+GSTP1-1), as percentage RFU ± SD ($n = 2$) compared to the treated control (C+C). Statistical analysis performed by two-sided unpaired *t*-test; $*P < 0.05$, $**P < 0.01$.

Clozapine toxicity in yeast is unrelated to mitochondrial dysfunction

Formation of ROS and inhibition of growth induced by the non-steroidal inflammatory drug diclofenac has previously been shown to be mediated by mitochondrial dysfunction by using respiratory deficient (rho^0) yeast cells (*25*). In this study, a similar strain was transformed with the BM3 M11 and control plasmid and treated with clozapine to investigate a possible involvement of the mitochondria in clozapine toxicity. Both the wild type and rho^0 strains transformed with the empty control plasmid showed an equal inhibition of growth compared to untreated controls, as shown in Fig. 9a. Expression of BM3 M11 resulted in a significantly increased toxicity, to the same extent in both strains. Moreover, Clozapine-induced ROS formation was similar in the wild type and rho^0 cells (Fig. 9b). Together these results suggest that clozapine toxicity in yeast is unrelated to mitochondrial electron transport chain (mETC) activity.

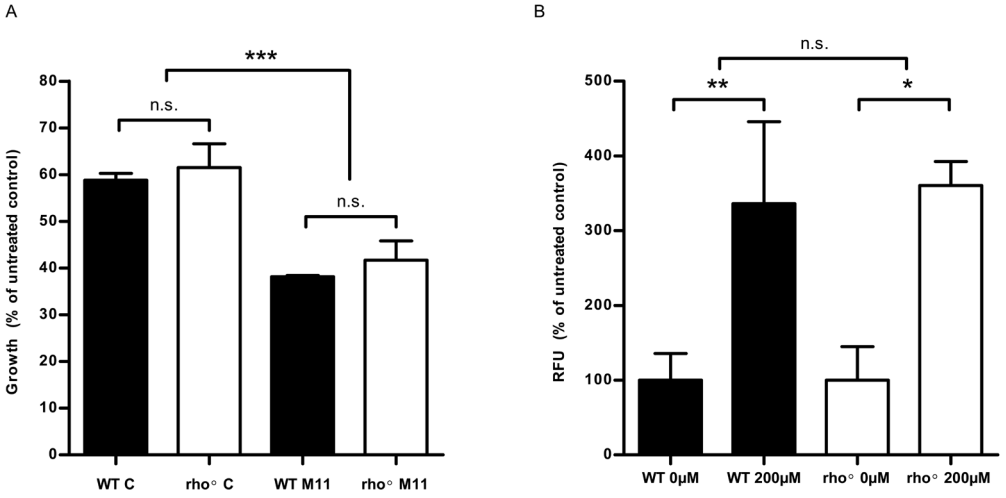

Fig. 9. Clozapine (200 µM) induced growth inhibition and ROS formation in wild type (WT, black bars) and respiratory deficient (rho^0, white bars) yeast. (A) Growth inhibition of strains without (C) and with BM3 M11 expression (M11) by 21 h incubation with clozapine. Growth is expressed as percentage growth ± SD (n = 2) compared to untreated cells. (B) Relative ROS formation after 2 h incubation with clozapine in WT and rho^0 strains without expression of BM3 M11. ROS formation is expressed as percentage RFU ± SD (n = 2) compared to untreated cells. Statistical analysis performed by two-sided unpaired t-test; n.s. not significant, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

DISCUSSION

The formation of reactive metabolites is an often-proposed explanation for the development of adverse drug reactions in clozapine treatment. In our study, we used yeast to investigate both the involvement of CYP bioactivation of clozapine on cellular toxicity, as well as the influence of GST catalyzed detoxification of the clozapine nitrenium ion, the proposed reactive metabolite of clozapine. By using the engineered M11 mutant of the bacterial CYP BM3, we showed the formation of human relevant clozapine metabolites in yeast, as is reviewed in supplementary Table S1. Although the parent compound already caused inhibition of growth and formation of ROS at 100 µM, at higher clozapine concentrations oxidative metabolism of clozapine by BM3 M11 further increased both

adverse events. This confirms the work in previous studies that showed a rise in toxicity depending on oxidative metabolism of clozapine in mammalian cellular models (*11*, *13–15*), indicating a role for the reactive nitrenium ion of clozapine in toxicity. The increased toxicity is most likely unrelated to the formation of the two major stable metabolites, N-desmethylclozapine and clozapine-N-oxide, as both of these were not more toxic to yeast than clozapine itself, like previously reported by (*11*) in human neutrophils. The substantial toxicity already caused by clozapine itself in yeast lacking the bioactivation by BM3 M11 remains unexplained, but might be related to an as yet to be identified off-target. Also in neutrophils, clozapine has been shown to cause apoptosis by a metabolism-independent mechanism (*11*).

To study the detoxifying potential of GST catalyzed GSH conjugation of the clozapine nitrenium ion in a eukaryotic model cell, we created yeast strains co-expressing BM3 M11 with different hGSTs. Of the three isoforms tested (A1-1, M1-1, P1-1), only hGSTP1-1 significantly protected against the BM3 M11 dependent inhibition of growth by clozapine. Previous work showed hGSTP1-1 to be more active towards the clozapine nitrenium ion than hGSTA1-1 and hGSTM1-1 (*17*). This higher catalytic activity could possibly explain the observed protection by hGSTP1-1 and in contrast not by hGSTA1-1 or hGSTM1-1 at the equal expression levels in our yeast strains. Absolute quantification of the conjugates based on the LC–MS data would require the use of analytical standards that were not available in this study. However, by assuming similar extinction coefficients and ionization for the different conjugates, there are not clearly increased levels of the sum of all conjugates in the hGSTA1-1, hGSTM1-1 or hGSTP1-1 strains based on the integration of the UV and EIC chromatograms. This would then suggest that the isoform-specific metabolic profile of clozapine conjugates is responsible for the difference in growth inhibition, rather than increased total conjugation.

hGSTP1-1 differs from hGSTA1-1 an hGSTM1-1, because it mainly catalyses the formation of C-8 glutathionyl deschloroclozapine (CG-6). As a result, the formation of the spontaneous C-6 glutathionyl clozapine (CG-1) and C-9 glutathionyl clozapine (CG-3) is decreased, as well as their corresponding cysteine conjugates. This shift in conjugation from mainly CG-1 and CG-3 to CG-6 is much less pronounced for hGSTA1-1 and hGSTM1-1. Possibly the chlorine substitution in CG-6 prevents interaction of the clozapine-derivative with the original clozapine off-target, while the other clozapine GSH- and cysteine conjugates still contain the chlorine group that might be required for their binding to a cellular off-target. Moreover, some of the GSH conjugates seem to be readily degraded to the corresponding cysteine conjugates in yeast (especially CG-6), whereas others are not (particularly CG-3). The cysteine conjugates are present at considerable levels compared to the GSH conjugates, underscoring the importance to take these into account as well. Previously, uncompromised vesicle mediated transport was associated with the resistance towards clozapine in yeast (*36*). This suggests involvement of the vacuole, known to be crucial in the degradation of GSH conjugates of xenobiotic electrophiles in yeast (*37*). Most of these genes involved in the vesicle transport have close human homologs, stressing the possible importance of this observed relationship in man (*36*). Thus, isoform-specific GSH conjugates of clozapine may differ in their susceptibility towards degradation and in their toxicity.

The human relevance of the potential contribution of hGSTP1-1 to the protection in clozapine toxicity will depend on relative expression levels and tissue distribution of GST isoforms. In human liver GSTP1-1 is expressed at much lower levels compared to GSTA1-1 and GSTM1-1 (*38*), and it is therefore questionable if hGSTP1-1 plays a role in the protection against clozapine-induced liver toxicity. However, hGSTP1-1 is more widely distributed throughout the body than most other isoforms (*39*). In some tissues, especially brain, lung and heart, hGSTP1-1 is the predominant isoform. Likewise, it is interesting to note that hGSTP1-1 is the most abundant GST isoform in many hematopoietic cell lines and in hematopoietic stem cells derived from human foetal liver (*40*, *41*). Therefore, hGSTP1-1 might be more important in the protection against clozapine-induced agranulocytosis. Four alleles of the hGSTP1-1 isoform have been identified in the human population that show differences in their catalytic activity for several substrates (*16*). It remains to be seen if these polymorphic variants affect the catalytic GSH conjugation of the clozapine nitrenium ion, and thereby contributes to the interindividual differences in the susceptibility to clozapine ADRs.

Several studies reported increased ROS formation upon clozapine treatment in mammalian cells (*15*, *35*, *42*). ROS production can result in oxidation of proteins, of which several have been identified in clozapine treated neuroblastoma cells, their functions mainly related to energy metabolism (*35*). Clozapine-induced ROS production has also been observed in our yeast model. However our results indicate that there is no clear correlation between ROS formation and growth inhibition caused by clozapine. hGSTP1-1 does not lower intracellular ROS levels although it protects from BM3 M11-dependent inhibition of growth. Clozapine-N-oxide does not inhibit growth while showing clear production of ROS. Yeast has previously successfully been used as a model to study diclofenac-induced dysfunction of mitochondria, a common source of ROS (*25*). Using a similar strategy, we showed that clozapine-induced ROS is not originating from the mETC. Clozapine treatment of respiratory deficient strains resulted in the same decrease in growth as observed in the wild type, both without and with bioactivation. Together, these results rule out involvement of the mETC in clozapine toxicity in yeast, which is in line with previously published results in mammalian cells (*14*, *43*).

We present, to our knowledge for the first time, a model combining CYP bioactivation with hGST catalyzed conjugation of drugs in yeast. Expression of hGSTA1-1 and hGSTP1-1 in yeast has been shown to protect against several antitumor drugs, but this study did not include CYP bioactivation (*44*). Recently the use of humanized yeast to study metabolism-related toxicity of drugs was reviewed (*19*). Co-expressions of phase I and phase II metabolic enzymes have been performed in yeast before, such as the CYP BM3 M11 mutant with human SULT1A1 (J.S. van Leeuwen, D.M. Vredenburg-Maasdijk, unpublished results), and rat CYP1A1 together with UGT1A6 (*45*), but their use in drug toxicity studies has not been reported. We show that such co-expressions in yeast offer a cellular model to study the effects of biotransformation on drug toxicity. Apart from this application, this model might be used to synthesize complex drug metabolites by whole cell biocatalysis, for example to produce regioselective GSH conjugates on a large scale to facilitate their structural elucidation or to study the toxicity of such metabolites.

CONCLUSION

We demonstrated that the yeast *Saccharomyces cerevisiae* could be manipulated to serve as a novel cellular model to study the interplay and effects of combined CYP and GST metabolism on drug toxicity. Using this model, we have shown a CYP dependent increase in clozapine toxicity, and subsequent isoform-specific protection by human GSTP1-1, in contrast to hGSTA1-1 and hGSTM1-1. Clozapine-induced formation of ROS was not directly linked to the inhibition of growth, and the mETC was not involved. Protection of hGSTP1-1 against the increased toxicity by CYP metabolism is most likely caused by increased GSH conjugation of the clozapine nitrenium ion or differential toxicity of the GST isoform-specific GSH conjugates, of which some are more readily degraded to their corresponding cysteine-conjugates in yeast than others.

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SUPPLEMENTARY DATA

Table S1. Clozapine metabolites identified in this study and human relevancy

Metabolite	Previous identification reported in literature
Clozapine-N-oxide (C-1)	Human urine (*31*)
N-desmethylclozapine (C-2)	Human urine (*31*)
C-6 glutathionyl clozapine (CG-1)	HLM[a] (*22; 17*)
	Rats and mice bile (*9*, conjugate 3c)
	Human neutrophils (*8*)
C-9 glutathionyl clozapine (CG-3)	HLM (*22; 17*)
	Rats and mice bile (*9*, conjugate 3a)
	Human neutrophils (*8*)
C-7 glutathionyl clozapine (CG-4)	HLM (*22; 17*)
	Rats and mice bile (*9*, conjugate 3b)
	C-7 methylthio clozapine[b] in human urine (*30*)
C1-4 glutathionyl clozapine (CG-5)	HLM with hGSTA1-1 and P1-1 (*17*)
	Rats and mice bile (*9*, conjugate 3d)
C-8 glutathionyl deschloroclozapine (CG-6)	HLM with hGSTA1-1, M1-1 and P1-1 (*17*)
	Rats and mice bile (*9*, conjugate 1)
	C-8 methylthio deschloroclozapine[c] in human urine (*46*)

[a] Human liver microsomes
[b] Likely product of the catabolism of CG-4
[c] Likely product of the catabolism of CG-6

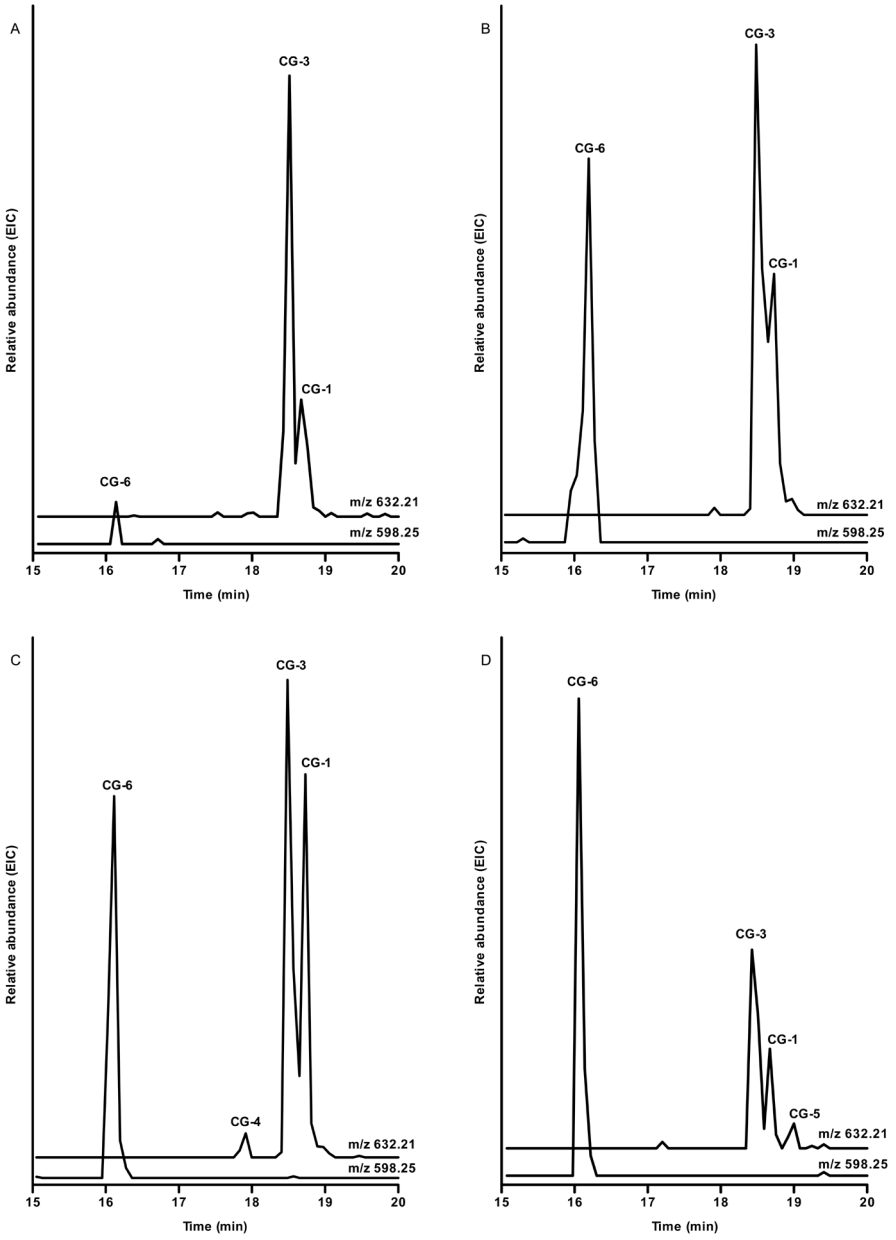

Fig. S1. Isoform-specificity of the GST-catalysed GSH conjugates in yeast strains co-expressing BM3 M11 with hGSTs. Shown are LC-MS extracted ion chromatograms (EIC) of yeast cellular extracts of cells incubated with 200µM clozapine, relative to the most abundant GSH conjugate in each sample. Strains expressed BM3 M11 either without (A) or combined with hGSTA1-1 (B), hGSTM1-1 (C) or with hGSTP1-1 (D). CG-1: C-6 glutathionyl clozapine; CG-3: C-9 glutathionyl clozapine; CG-4: C-7 glutathionyl clozapine; CG-5: C1-4 glutathionyl clozapine; CG-6: C-8 glutathionyl deschloroclozapine.

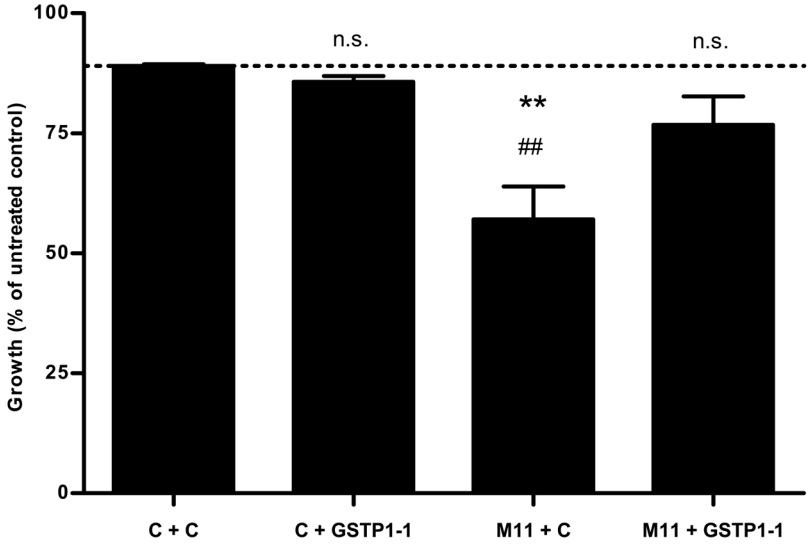

Fig. S2. Growth inhibition of the control strain (C+C), the strain expressing BM3 M11 without hGST (M11+C), the strain expressing hGSTP1-1 without BM3 M11 (C+GSTP1-1) and the strain co-expressing BM3 M11 with hGSTP1-1 (M11+GSTP1-1) after 72-hour incubation with clozapine (200µM). Growth is expressed as percentage growth ± SD (n=2) compared to untreated cells. Statistical analysis performed by two-sided unpaired t-test; n.s. not significant, **P<0.01, compared to C+C; ## P<0.01, compared to M11+GSTP1-1.

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